A disulfide reductase in spores of *Bacillus cereus* T

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An enzyme obtained from *Bacillus cereus* T spores which catalyzes the reduction of the disulfide, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), has been partially purified and characterized. The enzyme required either reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) or reduced nicotinamide adenine dinucleotide ($NADH_2$) as electron donor. It had a pH optimum of 8, was destroyed by heating at 70C for 5 min, and was stimulated by Ca^{2+} and Mg^{2+}. No other small molecular weight disulfides were found to be substrates for the enzyme.

Introduction

The biological reduction of disulfides in nature is well known. Numerous examples have been reported in the literature, including yeast and pea seed cystine reductase (13), glutathione reductases of mammalian and microbial origin (12, 14), and reductases which reduce dithio containing proteins involved in methionine sulfoxide reduction (2), oxidative decarboxylation of glycine (1), and reduction of cytidine diphosphate (10).

During a previous study of the thiol and disulfide content of dormant and germinating spores (4) we obtained data indicating that an increase in thiol groups occurred during germination. This suggested the possible existence of a spore enzyme capable of reducing disulfide bonds and led us to search for such an enzyme. Subsequently, a preliminary report from this laboratory (3) was given describing disulfide reducing activity in crude spore extracts of several *Bacillus* species and a partial characterization of the enzyme obtained from *B. megaterium*. This paper describes, in more detail, the purification and characterization of a similar enzyme from *B. cereus* T spores and discusses its possible significance in the spore, taking into account the more recent data of Setlow and Kornberg (18) concerning spore thiols and disulfides.

Materials and Methods[2]

Organism and Cultural Conditions

Spores of *B. cereus* T QMB 1590 obtained from H. L. Levinson, Pioneering Research Division, U.S. Army Natick Laboratories, Natick, Mass., were produced in G-medium as described by Hashimoto *et al.* (7). Harvested spores were cleaned in the polyethylene glycol–phosphate buffer (Y) system described by Sacks (15), thoroughly washed with cold demineralized water, and freeze-dried.

Preparation of Extracts

Spores were ruptured by sonicating spore (2 g)–glass bead (30 g) mixtures (No. 380-5005 Superbrite glass beads, Minnesota Mining and Manufacturing Co., St. Paul, Minnesota) suspended in 20 ml 0.05 *M* phosphate buffer, pH 8.0. Spore rupture was accomplished with a Branson Sonifier (model S-75, Branson Instruments Inc., Danbury, Connecticut) operating at 7–8 A for a total of 8 min sonification, by alternately sonicating and cooling the mixtures for 2-min intervals in an ice bath. Beads and debris were removed by centrifuging at $30\,000 \times g$ at 1–3C. Extracts thus obtained were stored in liquid nitrogen.

Purification Procedures

Crude extracts were diluted to about 10 mg protein per milliliter and nucleic acids partially removed by precipitation with streptomycin sulfate (final concentration 1.5% w/v). The clear supernatant resulting after centrifugation was sequentially fractionated with ammonium sulfate. The active fraction was dialyzed overnight and a 65-mg protein portion was chromatographed on a 2.5×8.0 cm Cellex-D (Bio-Rad Laboratories, Richmond, California) column equilibrated with 0.02 *M* phosphate buffer, pH 7.0. After protein application the column was eluted stepwise with two column volumes each of 0.02 *M*, 0.10 *M*, and 0.14 *M* phosphate buffer, pH 7.0, followed by a linear concentration gradient elution consisting of 150 ml each of 0.16 *M* and 0.30 *M* phosphate buffer, pH 7.0. Active fractions were pooled, concentrated by ultrafiltration (using an Amicon ultrafiltration cell with PM-30 membrane), and stored in liquid nitrogen. All manipulations were performed at 0–3C.

Enzyme Assay

The standard reaction mixture contained 150 μmoles phosphate buffer, pH 8.0; 2.1 μmoles 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB); 2 μmoles reduced nicotinamide dinucleotide phosphate ($NADPH_2$); enzyme; and water to a final volume of 3.0 ml. Disulfide reductase

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[2]Mention of brand or firm name does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

TABLE 1

Purification procedure for *Bacillus cereus* T disulfide reductase

	Protein recovered, mg	Total units	Specific activity, $\times 10^3$	Purification	Recovery, %
Crude extract	2110	11.9	5.6	1	
Ammonium sulfate 70–90% saturation	129	5.6	43.9	7.8	47
Cellex-D	3.1	2.4	540	96	20

activity was measured spectrophotometrically by observing the increase in absorbance at 412 nm caused by thionitrobenzoic acid (E_m = 13 600 at 412 nm) (5) formed as a result of DTNB reduction. All assays were conducted at room temperature using a Beckman DU-2 spectrophotometer. Measurements were made over the first 4 min incubation during which time absorbance changes of 0.005 to 0.015 per minute could be used. Absorbance of the thionitrobenzoic acid was linear over the range of 1 to 100 nmoles. All assays were performed by diluting enzyme as required to fall within these conditions. Reaction mixtures omitting either $NADPH_2$ or protein were included to permit correction for non-enzymatic reduction.

A unit was defined as that quantity of enzyme producing 1 μmole —SH per minute under the conditions described above. Specific activity was taken as the number of units per milligram of protein.

Analytical Methods

Protein was determined either by the biuret method (6) or spectrophotometrically (8).

Analytical acrylamide gel disc electrophoresis was performed in 7.5% gel using Tris-glycine buffer (pH 8.3) in the Canalco system (Canal Industrial Corporation, Rockville, Md.) at 1–3C.

$NADH_2$, $NADPH_2$, and DTNB were products of Calbiochem, Los Angeles, Calif.

Results

Enzyme Purification

Results of the purification procedure are summarized in Table 1. Most of the DTNB-reducing activity was precipitated between 70 to 90% saturation ammonium sulfate. This fraction contained a substantial amount of nondialyzable material with high absorbance at 270 nm. DTNB-reducing activity was eluted from the Cellex-D column during the concentration gradient portion of elution between 0.18 *M* and 0.20 *M* phosphate buffer.

Analytical acrylamide gel disc electrophoresis of pooled and concentrated Cellex-D purified fractions revealed the presence of one major and two minor components (see Fig. 1). When parallel, unstained gels were incubated in a standard reaction mixture immediately after electrophoresis, a yellow band developed which corresponded exactly with the major component of stained gels.

The results of several experiments served to confirm the enzymatic nature of DTNB reduction by the purified enzyme. DTNB reduction was found to be linearly proportional to protein concentration between about 1 to 5 μg purified enzyme using standard reaction conditions (as

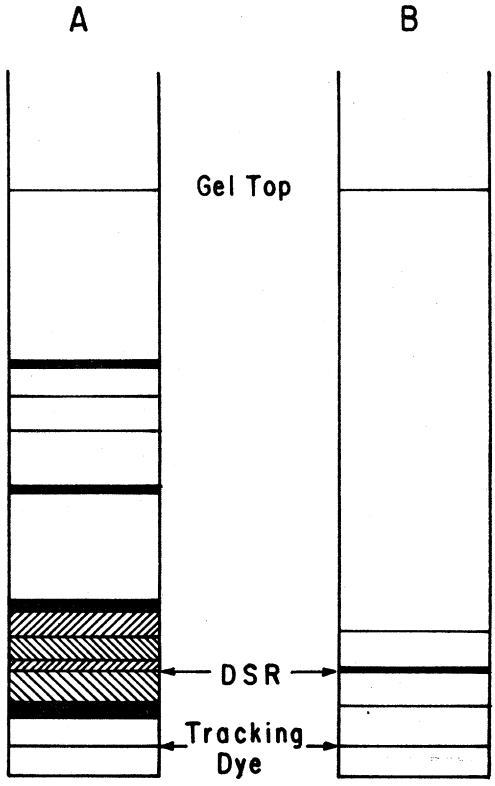

FIG. 1. Acrylamide gel disc electrophoresis of *Bacillus cereus* T disulfide reductase (DSR): (A) 70–90% saturation ammonium sulfate fraction (100 μg protein); (B) Cellex-D fraction (75 μg protein).

shown in Fig. 2). Values in tables and figures were correlated for nonenzymatic DTNB reduction.

Thermal stability experiments were performed in screwcap tubes using dialyzed purified enzyme preparations diluted with water. Heating was accomplished by submerging tubes in a constant-temperature water bath for 5 min at various temperatures. The desired temperatures were controlled to a precision of ± 0.1 degree. After heating, tubes were quickly cooled in tap water before addition of the remaining reaction components. The enzyme was found to possess no unusual heat stability characteristics, as can be seen in Fig. 4.

The pH optimum of the enzyme was determined using phosphate, tris(hydroxymethyl) aminomethane chloride, and glycine–NaOH buffers over the range of 6 to 10. After incubation, reaction mixtures were immediately chilled and held in an ice bath while the pH was adjusted to 8.0 with cold 1 *N* NaOH or HCl and brought to a volume of 4.0 ml with cold water. Absorbance of reaction mixtures was then quickly measured while the mixtures were cold. The absorbance of reaction mixtures at pH 8.0 after 4 min incubation was compared with that of similar reaction mixtures that were immediately chilled after incubation. Absorbance was found to be the same in both cases, indicating that no significant additional reaction occurred after or during chilling. This procedure was followed to determine the thionitrobenzoic acid reduction product which absorbs maximally between pH 8 and 9 (17). The results indicated the optimum to be about pH 8.0.

A comparison of electron donors revealed that both $NADH_2$ and $NADPH_2$ could serve for DTNB reduction. The rate of reduction with

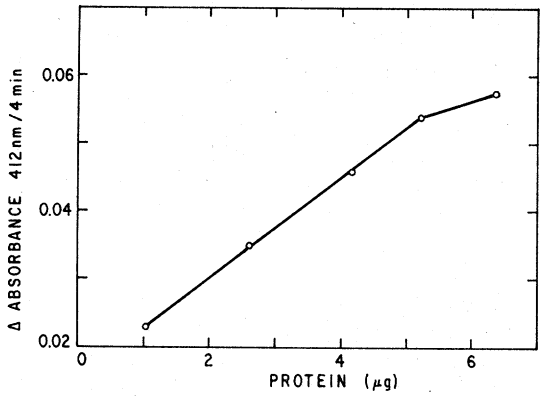

FIG. 2. Effect of protein concentration on reduction of 5,5′-dithiobis(2-nitrobenzoic acid) by Cellex-D purified *Bacillus cereus* T disulfide reductase. See Materials and Methods for standard reaction conditions.

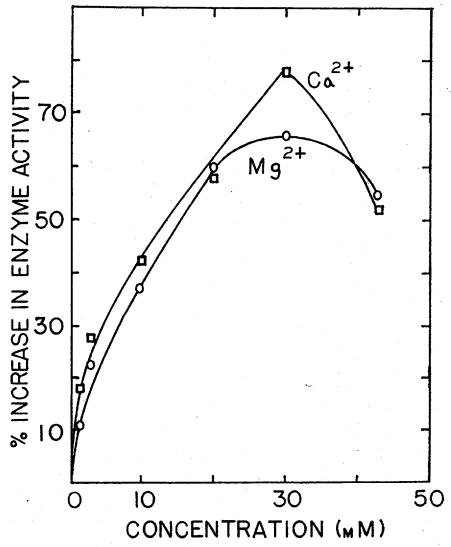

FIG. 3. Stimulation of *Bacillus cereus* T disulfide reductase by calcium and magnesium. See Materials and Methods for standard reaction conditions.

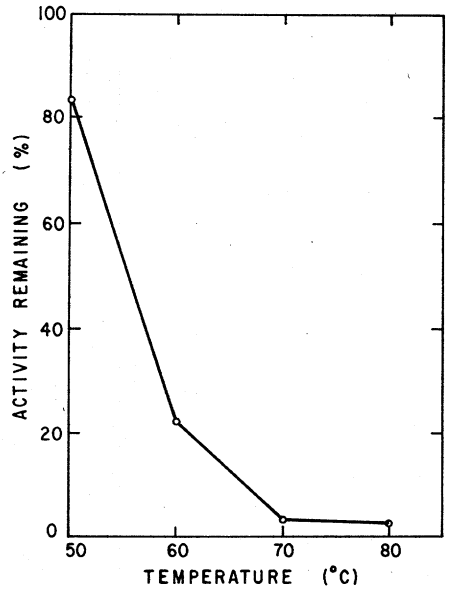

FIG. 4. Heat stability of Cellex-D purified *Bacillus cereus* T disulfide reductase. Heating time was 5 min. Protein concentration was 5 μg.

$NADPH_2$ (3.45 nmoles —SH per minute) was 1.7 times greater than with $NADH_2$ (2.07 nmoles per minute).

Several divalent and monovalent cations were tested for their capacity to stimulate or inhibit the disulfide reductase (DSR). Figure 3 illustrates the stimulation observed with calcium and magnesium. Manganese, copper, sodium, and potassium were found to be inhibitory.

A variety of compounds were tested to determine substrate specificity of the enzyme. These included L-cystine, DL-methionine, DL-homocystine, oxidized lipoic acid, DL-methionine sulfoxide, oxidized glutathione, and 2-hydroxyethyl disulfide. In these experiments, the above potential substrates were substituted for DTNB in the standard reaction mixture at 10 μmole concentration. Enzyme activity was measured spectrophotometrically by following the decrease in absorbance at 340 nm. $NADPH_2$ concentration was reduced sufficiently to fall within the spectrophotometer detection capability. Incubation periods were extended to 20 min to detect slow reactions. This type assay was possible because purified enzyme preparations were devoid of $NADH_2$ oxidase activity. No substrate-dependent oxidation of $NADPH_2$ was observed with any of the compounds tested.

Thiol-disulfide transhydrogenase activity of purified spore disulfide reductase was tested according to the assay procedure used by Nagai and Black (11) for their yeast transhydrogenase. In these experiments reduced glutathione served as electron donor and either L-cystine or 2-hydroxyethyl disulfide as the acceptor. No thiol-disulfide transhydrogenase activity was detected with the electron donor–acceptor pairs tested.

Discussion

We have previously demonstrated the existence of disulfide-reducing activity in crude extracts obtained from several *Bacillus* sp. spores (3). Purification and determination of some characteristics of the enzymes from *B. megaterium* and *B. cereus* T spores also has been accomplished and the properties of these two enzymes are quite similar. Classification of these enzymes, however, has been a special problem because the only substrate presently known is an artificial, non-biological compound, DTNB. In the case of *B. cereus* T spore DSR it can be reasonably concluded, on the basis of substrate specificity experiments, that spore DSR by itself is not a glutathione reductase (12), cystine reductase (13), oxidized lipoic acid reductase (9), or methionine sulfoxide reductase (2). Also it is probably not a reduced glutathione-disulfide transhydrogenase similar to the yeast enzyme (11), but other transhydrogenase activity cannot be completely ruled out because of the numerous electron donor–acceptor pairs available. It must also be acknowledged that the number and variety of known enzymes or enzyme systems capable of reducing DTNB using $NADH_2$ or $NADPH_2$ as electron donors is largely unknown and data are not available in the literature on many reductase type enzymes. Consequently, for the present, the natural substrate of the spore enzyme remains obscure and it temporarily could be referred to as "reduced NADP: DTNB oxidoreductase" or "DTNB disulfide reductase."

It has been necessary to modify our initial ideas concerning the appearance of thiol groups during germination with the report of Setlow and Kornberg (18), in which it was demonstrated that no net change in thio–disulfide ratio occurred during germination. However, an enzymatically catalyzed thio–disulfide interchange remains an interesting possibility in spore germination which would not require a substantial reducing power reservoir and would result in no net change in the thiol–disulfide ratio. It is worth noting that the ribonucleoprotein thiol–disulfide transhydrogenase reaction in sea urchin eggs described by Sakai (16) involves a contractile protein which contracts or relaxes, depending upon its state of thiol oxidation. If a similar reaction occurred during spore germination it is easy to imagine a relaxation of the disulfide-rich coat protein with a subsequent alteration in permeability.

Another equally attractive possibility is activation of thiol-type enzymes involved in germination which were theoretically protected during dormancy by disulfide bridging. Or, perhaps the substrate for spore DSR may not exist in the dormant spore, but is synthesized at some point during germination or outgrowth. We are hopeful that continuing studies will elucidate the true function of spore DSR.

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